Free Rad. Res. Comms., Vol. 9, No. 3-6, pp. 399-405
Reprints available directly from the publisher
Photocopying permitted by license only

PRINCIPLES OF THE METABOLISM OF NITROXIDES AND THEIR IMPLICATIONS FOR SPIN TRAPPING

HAROLD M. SWARTZ

University of Illinois College of Medicine at Urban-Champaign, 506 S Mathews, Urbana, Illinois 61801, USA

A review of the principal interactions of nitroxides with cells suggests that if these same phenomena occur with spin adducts the result could be considerable experimental confusion and error. In particular, these could lead to differential rates of loss of spin adducts, thereby potentially invalidating conclusions on the amounts or even the types of free radicals that are trapped. In addition, shuttling of electrons between nitroxides and hydroxylamines also very significantly could alter the amounts and types of spin adducts that are observed.

KEY WORDS: Nitroxides, spin adducts, metabolism, hydroxylamines, electron shuttling.

INTRODUCTION

The metabolism of nitroxides by functional biological materials can be a critically important factor that can affect their usefulness and can greatly alter the interpretation of data obtained with them. When properly understood, the metabolism can be exploited to obtain additional information that may not be obtainable by other means while if the metabolism is not taken into account, serious errors can result from the uses of nitroxides in biological systems. When a spin trap reacts with a radical, the product usually is a nitroxide and therefore results of studies of the metabolism of nitroxides also may apply to the metabolism of spin adducts.

Recently, nitroxides have been used increasingly in viable biological systems, both for their traditional uses as spin labels and for newer and potentially very valuable uses as probes of oxygen concentration, as probes of redox metabolism, as the active agents for ESR imaging and ESR spectroscopy in extended objects, and as contrast agents for NMR imaging and NMR spectroscopy of biological systems. This increased use of nitroxides in functional biological systems has resulted in an increased number of studies aimed at understanding their metabolism and distribution in such systems. Although much remains to be learned about this subject, some useful general principles have emerged as a result of these investigations from a number of laboratories. In this paper some of these results are summarized very briefly as general principles of the metabolism of nitroxides. It is not yet clear to what extent these principles will apply to the metabolism of spin adducts but, at the minimum, the studies with nitroixdes provide evidence that there is urgent need to study similar questions in spin adducts because if the results from the studies of nitroxides do apply to spin adducts, these have significant implications for the interpretation of spin trapping studies. It should be noted that necessarily, the conclusions stated as "princi-

ples" are over simplified and the indicated references should be used as starting points for gathering more complete information. As a matter of convenience most of the references are to papers from the author's laboratory; the references within these papers provide more general access to the literature.

PRINCIPLES OF METABOLIC INTERACTIONS OF NITROXIDES WITH CELLS

1. Reduction of Nitroxides by Cells Occurs Almost Exclusively Intracellularly

This principle is indicated most clearly by the fact that in a suspension of cells nitroxides which have a permanent charge are not reduced rapidly unless the cell membranes are broken; then these nitroxides are reduced at rates that are consistent with the structural characteristics of the nitroxide.[1] In the intact animal there may be additional reduction in the extracellular compartments, primarily by ascorbate.

2. Reduction by Cells Occurs Primarily Through Enzyme Associated Mechanisms

This is indicated by a variety of evidence, especially the loss of the ability of cells to reduce nitroxides when subjected to treatments that inactivate enzymes and by the relationship of the rates of reduction to temperature.[2,3]

3. Rates of Reduction of Some Nitroxides by Cells are Sensitive to the Concentration of Oxygen

This is indicated by the observed rates of reduction in the presence and absence of oxygen, with the rates for many nitroxides increasing in the absence of oxygen.[1,4,5] This increase can be as high as 40-fold (for 5-doxyl stearate). Some nitroxides, however, have rates of reduction that are independent of the concentration of oxygen.

4. The Full Effect of the Suppression of the Rate of Reduction of Nitroxides by Oxygen Occurs with Low Levels of Oxygen (about one micromole)

Increasing the oxygen concentration above 1 micromole has no further effect on the rate of reduction of the nitroxides.[5] This effect of oxygen occurs via a decrease in the rate of reduction rather than an increase in the reverse reaction (but see also principle 18).

5. The Principal Site of Reduction of Nitroxides is in the Mitochondria

This is a tentative conclusion, requiring additional data to confirm whether this applies to all nitroxides. It is based on similarities between factors that affect the reduction of nitroxides in isolated mitochondria and in whole cells.[2,3] This includes, especially, the effects of inhibitors and the effects of oxygen. It appears that the site of reduction in mitochondria may be at the level of ubiquinone in the electron transport chain.

6. The Microsomal Fraction of Cells can Metabolize Nitroxides

This is based on studies with isolated microsomal fractions.[6,7] There appears to be activity associated with both NADH and NADPH dependent systems.

7. The Cytoplasmic Fraction of Cells has Very Low Reducing Activity for Nitroxides Unless There Are Substantial Levels of Ascorbate Present

This is based on studies of cytoplasmic fractions isolated from cells.[8] There are high levels of ascorbate in the cytoplasm of hepatocytes and these can lead to experimental confusion because of the fragility of isolated hepatocytes, which results in leakage of ascorbate into the extracellular medium.[9]

8. The Type of Ring is the Principal Structural Variable that Affects the Rate of Reduction of Nitroxides

This is based on studies in both model systems and functional biological systems. Nitroxides based on the 6-membered piperidine ring usually are reduced faster than comparable nitroxides based on the 5-membered pyrroline or pyrrolidine rings.[1,9,10] Although direct structural analogs based on doxyl rings are sparse, this type of nitroxide appears to be relatively susceptible to reduction. The different rates of reduction with the different rings may be due to the accessibility of the nitroxide group rather than differences in redox potential.[10]

9. The Type of Substituent on a Particular Ring System Can Have a Predictable Effect on Rates of Reduction

This is based on a comparison of electrochemical potentials with the rate of reaction with ascorbate, for a limited series of nitroxides.[10]

10. Differences in the Location of Nitroxides May Alter Their Reactivity Significantly

This is illustrated in the different rate of reduction of doxyl stearates which differ only in regard to the location of the nitroxide function on the hydrocarbon chain of these fatty acid analogs.[3] In general, the degree of lipophilicity or the presence of a permanent charge will significantly affect the location of the nitroxide and its rate of reduction in complex biological systems.

11. The Principal Products of the Metabolism of Nitroxides are the Corresponding Hydroxylamines

This is based on the finding that the use of appropriate mild oxidants that are capable of oxidizing hydroxylamines to nitroxides but cannot oxidize amines to hydroxylamines or nitrones, can restore essentially all of the original nitroxide after bioreduction.[5,11] There is little evidence for the oxidation of nitroxides or their reduction below the level of hydroxylamines.

12. Hydroxylamines and Nitroxides can Shuttle Electrons Readily, Oxidizing the Hydroxylamines to Nitroxides and Reducing Nitroxides to Hydroxylamines

This now has been demonstrated in a variety of systems with a variety of nitroxides and can, potentially, lead to experimental confusion when there are two or more types of nitroxides/hydroxylamines present and/or there are two or more compartments in the system.[12]

13. Relative Rates of Reduction of Nitroxides in Model Systems May Not Necessarily be Reflected in Functioning Biological Systems

These effects can be fairly obvious, e.g., in the situation in which the nitroxide has a positive charge and therefore cannot enter into the interior of cells where the reducing mechanisms are located. These effects, however, also may be more subtle, with the relative rates of reactivity in the biological system not following the expected relationship in the biological system for reasons that are not readily obvious.[13]

14. Oxidation of Hydroxylamines Back to Nitroxides Can Occur at Very Significant Rates

This is based on studies which have followed the rates of formation of nitroxides from hydroxylamines in cells or subcellular fractions.[5,11,14] With some nitroxides, the rate of oxidation of the hydroxylamine can be comparable to the rate of reduction of the nitroxide.

15. For Those Hydroxylamines with a High Rate of Oxidation, the Principal Reaction Involves Enzymes

This has been shown by the effects of treatments that inactivate or alter the rates of enzymatic processes.[5,11,14]

16. The Enzymatic Oxidation of Hyroxylamines Occurs Principally in Membranes

This is indicated by the observation that only lipophilic hydroxylamines have significant enzymatically driven rates of oxidation.[14]

17. The Mechanism of Oxidation of Hydroxylamines Probably Involves Cytochrome Oxidase

This is indicated by studies with inhibitors of enzymes. Because the usual active site of cytochrome oxidase is in a hydrophilic region, this implies that a different part of the enzyme may be involved in the oxidation of hyroxylamines.[14]

18. The Rate of Oxidation of Hydroxylamines is Related to the Concentration of Oxygen

This has been demonstrated directly. The relationship between the rate of oxidation and the concentration of oxygen is quite different from that observed for the reduction of nitroxides. The rate of oxidation increases with increasing concentrations of oxygen at least up to concentrations of oxygen of 150 micromolar.[5]

19. The Metabolism of Lipophilic and Hydrophilic Nitroxides/Hydroxylamines is Significantly Different

This is clearly the case for the hydroxylamines because only the lipophilic nitroxides undergo enzymatic oxidation. It also appears to be true for the reduction of nitroxides: the principal sites and mechanisms of reduction may differ for hydrophilic and lipophilic nitroxides.

20. The Metabolism of Nitroxides/Hydroxylamines Probably Differs in Different Types of Cells

It has been shown directly by studies of several different types of cells.[8] This is an expected result because different cell lines can have different relative amounts of redox active substances and redox enzymes.

21. The Metabolism of Nitroxides/Hydroxylamines in Any Particular Cell Line May Vary Significantly with Changes in the Physiological State and Environment of the Cell

This has been observed directly and is expected because of the sensitivity of the metabolism of nitroxides/hydroxylamines to factors such as the redox state of the cell, the concentration of oxygen, etc.

22. A High Degree of Lipophilicity Will Alter the Localization and Reactivity of Nitroxides/Hyroxylamines Very Significantly

In particular, because most cells have relatively small amounts of lipids and these occur primarily in membranes, nitroxides that partition strongly into lipids will be concentrated into a small volume of the cells and will encounter a different set of enzymes than the hydrophilic nitroxides.[13,15]

23. The Rate Equations for Reactions of Nitroxides of Hydroxylamines Will be Complex

This is because of the occurrence of enzymatic and nonenzymatic mechanisms of reduction of nitroxides and oxidation of hydroxylamines and, probably, the occurrence of significant enzymatic reactions in more than one set of enzymes.[3]

INTERACTIONS OF SPIN ADDUCTS WITH CELLS

Only limited amounts of direct data are available on the interactions of spin adducts with cells. There have been direct studies of adducts of DMPO and DBNBS with hydroxyl radicals and superoxide anions.[16,17,18] These limited data indicate that in the presence of cells oxygen radical adducts of DMPO are not stable; cells will metabolize preformed DMPO adducts of hydroxyl radicals or superoxide anions that are added to suspensions of cells. DBNBS spin adducts with hydroxyl radicals or superoxide anions also appear to be unstable in cells. Indirect studies suggest that alkyl radical spin adducts of DMPO and PBN may be relatively stable inside cells. The loss of the spin adducts of DMPO in cells appears to be via metabolism into products that are

different than those observed with the metabolism of nitroxides, because ESR active species could not be restored by the use of a mild oxidant, ferricyanide, and the degradation of the spin adducts was not affected by processes that inactivate enzymes.

CONCLUSIONS

Because the products of radical trapping by spin traps are nitroxides, it is plausible to consider their interactions with cells in light of the available data on the interactions of nitroxides with cells. Such data indicate a number of areas of potential experimental concern because nitroxides are metabolized in cells at rates that vary with the type of nitroxide, the environmental conditions in the cell (especially the concentration of oxygen), and the type of cell. All of these factors would affect the amounts of spin adducts that would be observed and could affect different spin adducts differently.

Another factor of particular potential importance for spin trapping experiments is the finding that hydroxylamines and nitroxides can exchange electrons readily, thereby potentially decreasing the amount of nitroxide that is observed and/or generating ESR signals as hydroxylamines are converted to nitroxides. Many spin trapping experiments result in the generation of two or more types of spin adducts and there may be significant amounts of hydroxylamines in the original preparation of spin traps and more may be generated by reduction of the spin adducts. Such phenomena, together with the potential for differential rates of metabolism of the spin adducts could result in considerable experimental confusion.

It therefore seems essential that appropriate studies of the interactions of spin adducts with cells be carried out to determine if phenomena similar to those observed with nitroxides do occur with some or all of the usual spin adducts that are produced in studies of biological systems.

Acknowledgments

This summary is based on research supported by NIH grants GM 35534 and GM 34250 and used facilities supported by NIH grant RR 01811.

References

1. Swartz, H.M., Sentjurc, M. and Morse, P.D. II, Cellular Metabolism of Water-Soluble Nitroxides: Effect on Rate of Reduction of Cell/Nitroxide Ratio, Oxygen Concentrations, and Permeability of Nitroxides, *Biochem. Biophy. Acta*, **888**, 82–90, (1986).
2. Sentjurc, M., Morse, P.D., II, and Swartz, H.M. Influence of Metabolic Inhibitors on the Nitroxide Reduction in the Cells, *Periodic Biologicum*, **88**, 202–203, (1986).
3. Chen, K., Morse, P.D., II, and Swartz, H.M. Kinetics of Enzyme-Mediated Reduction of Lipid Soluble Nitroxide Spin Lables by Living Cells, *Biochem. Biophy. Acta*, **943**, 477–484, (1988).
4. Pals, M.A. and Swartz, H.M. Oxygen Metabolism of Potential MR Contrast Agents, *Invest. Radiol.*, **22**, 497–501, (1987).
5. Chen, K., Glockner, J.F., Morse, P.D., II, and Swartz, H.M. Effect of Oxygen on the Metabolism of Nitroxide Spin Labels in Cells, *Biochemistry*, **28**, 2496–2501, (1989).
6. Tomasi, A., Albano, E., Iannone, A.C., Swartz, H.M. and Vannini, V. Reduction Mechanism of the Spin Probe Tempol in Liver Microsomes, in *Medical, Biomedical and Chemical Aspects of Free Radicals* (Elsevier Science publish.), Amsterdam Press, (Netherland) (1988).
7. Iannone, A., Bini, A., Swartz, H.M., Tomasi, A. and Vannini, V. Metabolism in Rat Liver Microsomes of the Nitroxide Spin Probe, Tempol, Biochemical Pharmacology, in press (1989).
8. Unpublished data

9. Iannone, A., Hu, H., Tomasi, A., Vannini, V. and Swartz, H.M. Metabolism of Aqueous Soluble Nitroxides in Hepatocytes: Effects of Cell Integrity, Oxygen and Structure of Nitroxide, *Biochem. Biophy. Acta,* **991,** 90–96, (1989).
10. Morris, S., Swartz, H.M., Sosnovsky, G., Rao, N.U.M., Hui, B. and Huber, C.O. Chemical and Electrochemical Reduction Rates of Cyclic Nitroxides (Nitroxyls), submitted.
11. Chen, K. and Swartz H.M., The Products of the Reduction of Doxyl Stearates in Cells are Hydroxylamines as Shown by Oxidation by ^{15}N Perdeuterated Tempone, *Biochem. Biophy. Acta.* **992,** 131–133.
12. Nettleton, D.O., Morse, P.D. II, and Swartz, H.M. Exchange and Shuttling of Electrons by Nitroxide Spin Labels, *Arch. Biochem. Biophys.,* **271,** 414–423, (1989).
13. Sentjurc, M., Pecar, S., Chen, K. and Swartz, H.M. Cellular Metabolism of Proxyl Nitroxides and Hydroxylamines, submitted.
14. Chen, K. and Swartz, H.M. Oxidation of Hydroxylamines to Nitroxide Spin Labels in Living Cells, *Biochem. Biophys. Acta,* **970,** 270–277, (1988).
15. Nettleton, D.O., Morse, P.D., II, Dobrucki, J.W., Swartz, H.M. and Dodd, N.J.F. Distribution of 5-Doxylstearic Acid in the Membrane of Mammalian Cells, *Biochem. Biophys. Acta,* **944,** 315–320, (1988).
16. Samuni, A., Samuni, A. and Swartz, H.M. The Cellular-Induced Decay of DMPO Spin Adducts of ·OH and O_2^-., *Free Rad. Biol. Med.,* **6,** 179–183, (1989).
17. Samuni, A., Carmichael, A.J., Russo, A., Mitchell, J.B. and Riesz, P. On the Spin Trapping and ESR Detection of Oxygen-derived Radicals Generated Inside Cells, *Proc. Nat. Acad. Sci. USA,* **83,** 7593–7597, (1986).
18. Samuni, A., Samuni, A. and Swartz, H.M. Evaluation of Dibromo Nitrosobenzene Sulfonate as a Spin Trap in Biological Systems, *Free Rad. Biol. Med.,* **7,** 37–43, (1989).

Accepted by Prof. E.G. Janzen